GLIOTOXIN PRODUCTION IN ASPERGILLUS FUMIGATUS

Susan Musyimi

Gliotoxin Production in *Aspergillus Fumigatus*

By

Susan Musyimi

A Thesis Submitted in Partial Fulfillment of

Requirements of the CSU Honors Program

For Honors in the degree of

Bachelors of Science

in

Biology

College of Science

Columbus State University

Thesis Advisor _____ Date 5/1/08

Committee Member _____ Date 5/01/08

CSU Honors Program Director _____ Date 05/01/08

6-16-08

Abstract

Aspergillus fumigatus is a ubiquitous fungus that causes an infection called aspergillosis in immune-compromised surgery patients (Latge, 2001, 384; Bok et al. 2006). Aspergillosis cases have increased in the past decade due to the increasing number of patients undergoing immunosuppressive therapy and receiving organ transplants (Bok et al. 2006). Although there is still disagreement as to the causative agent of the symptoms accompanying aspergillosis, many scientists suspect that the compound, gliotoxin, plays a major role. In this study, we compared the amounts of gliotoxin produced from clinical and environmental isolates of *A. fumigatus*. We hypothesized that the amount of gliotoxin produced would be higher in the clinical isolates in comparison to the environmental isolates. Cell assay results showed that the clinical isolates produced more gliotoxin than the environmental isolates while the HPLC results showed no significant difference between gliotoxin production in clinical and environmental isolates.

Introduction

Aspergillus fumigatus is an opportunistic fungus that causes infections in immune-compromised surgery patients (Latge, 2001, 384). *Aspergillus fumigatus* causes an infection called aspergillosis (Bok et al. 2006). The incidence of Aspergillosis has increased in the past decade because of the increasing number of patients receiving organ transplants and immunosuppressive therapy (Bok et al. 2006). *A. fumigatus* is characterized as having a blue-green to grey colony coloration (doctorfungus). Compounds produced by *A. fumigatus* and suspected to be virulence factors are proteases and toxins produced by the fungi that might interfere with the lung epithelium (Latge, 2001, 384). Other molecules produced during the growth *A. fumigatus* that may aid in the mold's survival in the respiratory mucosa of the host include ribonuclease which causes host cell death, haemolysin which causes red blood cell lysis, and secondary metabolites such as gliotoxin which causes immunosuppression (Latge, 2001, 384). *Aspergillus fumigatus* establishes itself in the tissue of the patient to cause the illness.

The life cycle of *Aspergillus fumigatus* begins when asexual conidiophores are released into the environment (Bignell et. al 2004). Vegetative mycelium then give rise to more mycelia in the growing environment (Bignell et. al 2004). The features of *A. fumigatus* that increase its rate of infection are the small size of conidia, the large number of conidia produced from a single spore event, and the ability of the conidia to grow in different temperatures (Latge 2001). Also, there are no specific nutrient requirements for the growth of the mycelia (Latge 2001, 384). Therefore, there are various factors aiding in the survival of *Aspergillus fumigatus*.

To cause invasive aspergillosis, the conidia migrate to the alveoli and spread mycelia (Amitani et al. 1995). Amitani et al. (1995) found that clinical isolates of *A. fumigatus* slow ciliary beat frequency (CBF) by producing factors that damage cilia in vivo. Different body parts have different chances of infection and rates of cure and survival once infection has occurred (Singh 2005). Some parts of the

body show *A. fumigatus* infections late in the surgical recovery period while others have it early. Invasive aspergillosis mostly affects transplant recipients (Singh 2005).

In the lungs, *A. fumigatus* works by attacking the respiratory mucosa to cause complications due to production of toxic molecules. This type of infection is referred to as bronchopulmonary aspergillosis (Amitani et al. 1995). Singh (2005) found that single lung transplant patients were more likely to develop invasive aspergillosis if they had chronic obstructive pulmonary disease. The higher likelihood of aspergillosis might be because the spores are retained longer in the lung tissue, thus giving them time to establish their mycelia (Singh 2005). Also, invasive aspergillosis in single lung transplant recipients occurs later in the recovery period, and there is an increased likelihood of invasive aspergillosis compared to tracheobronchitis patients (Singh 2005). The mortality rate of single lung transplant patients is also higher than that of other lung transplant recipients (Singh 2005). Overall, the virulence factors are many but one that has received significant attention in the past decades is gliotoxin.

Gliotoxin is among a group of secondary metabolites that are suspected of causing immunosuppression. This group also includes fumagatin, fumagillin, helvoic acid, etc (Bok et al. 2006). Gliotoxin has been shown to have immunosuppressive effects by slowing the ciliary beat frequency (Amitani et al., 1995). Gliotoxin is immunosuppressive at relatively low concentrations of 0.03-1ug/ml. Gliotoxin first gained attention for its antibiotic qualities (Kaufmann et al. 2000). Despite its antimicrobial activity against fungus and bacteria, its immunosuppressive effects have prevented its clinical use (Tuch et al. 1988). Gliotoxin belongs to a group of compounds called epipolythiodioxopiperazines (Tuch et al. 1988). The reactive sulfur component of gliotoxin is thought to be responsible for its toxicity (Sutton et al. 1996). Gliotoxin inhibits phagocytosis, T-cell proliferation, cytolytic T lymphocytes activation, and adherence of macrophage, blood monocytes, fibroblasts and L929 cells (Kaufmann et al. 2000). Gliotoxin acts by inactivating antigen-presenting cells and facilitating

genomic destruction of such cells (Tuch et al. 1988). Gliotoxin may also cause immunosuppression through production of reactive oxygen species, inhibition of NF-*k*B and by inducing DNA damage through substrate cycling (Kaufmann et al. 2000).

In this study compared the amount of gliotoxin produced from with clinical and environmental isolates of *A. fumigatus*. We assumed that more gliotoxin production means that the strain is more virulent and likely to cause more sickness in the immune-compromised patient. We hypothesized that there will be more gliotoxin production in clinical isolates of *A. fumigatus* than in environmental strains of *A. fumigatus.*

Materials and Methods

Part 1. High Performance Liquid Chromatography

Chloroform extraction

The first step in extracting gliotoxin from *Aspergillus fumigatus* is culturing *Aspergillus fumigatus* on five Sabouraud agar plates for 2 days at 37°C. Secondly, we harvested the conidia using sterile 0.5% Tween 20. Then we adjusted the concentration of conidia $10^7 ml^{-1}$ in distilled water using a hemocytometer. Thirdly, we used 1ml of this conidial suspension to inoculate 100ml of Czapek-dox broth in a 250ml flask. Afterwards, we incubated cultures at 37°C in a shaking incubator at 1400 rpm for 2 days. Then, we harvested fungal biomass by filtration through Whatman N°1 filter paper in a Buchner funnel. We extracted the filtrate three times by shaking for 10 min with 50 ml chloroform at 25°C. We evaporated the extracts in the tubes to dryness by immersing in a water bath. We added 10 ml methanol in each of the tubes and store at -70°C until use.

Preparing the extracts for HPLC included diluting the gliotoxin extract 1/50 in 50% methanol 50% deionized-distilled water (the mobile phase) and injecting 10µl into the HPLC. Construct a standard

curve of peak height versus gliotoxin by injection of 20µl of gliotoxin standards (50, 100 and 200ngml^{-1} dissolved in the mobile phase) into the HPLC. To make the standard curve, we purchased synthetic gliotoxin and detected the lowest level at which gliotoxin could still be detected. We did this by diluting the gliotoxin and observing the absorbance (based on method by Richard *et al.* 1989). We performed five dilutions of synthetic gliotoxin (100ug/ml, 10ug/ml, 1ug/ml, 0.1ug/ml, 0.01ug/ml) in 70% methanol and ran them on an HPLC machine to determine the lowest amount that could still be detected. We made a graph of the area under the peak versus the concentration of the toxin. The procedure for HPLC analysis was adapted from a study by Richard et al. (1989). We used the equation from the calibration curve to find the amount of gliotoxin injected. The isolates were numbered but their identity was hidden to prevent a biased interpretation of the results.

Table 1. Dilutions of Gliotoxin

	Concentration (ug/ml)	**Injected (1ml into HPLC)**
	1000	10 ug
1:10	100	1 ug
1:20	50	0.5 ug
1:25	40	0.4 ug
1:50	20	0.2 ug

Part 2. Cell Assay

The L929 cells (mouse lung fibroblast cells) have previously been found to be detached from well plate by gliotoxin. We Cultured mouse lung fibroblast cells (L929) in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C for 2 days. We incubated the plates in 5% CO_2 for 2h at 37°C. We made a suspension of L929 cells that were now labeled with methyl dye. The cells were added to a 96 well plate. We incubated the cells for two hours.

After two hours we discarded the culture medium and washed the cell monolayers by adding excess PBS pH 7.3 at room temperature to the wells of the microtitre plate. We added culture extract to the cells. Some cells were detached from the plate while other remained attached. We removed the cells that were detached. Detergent Triton X-100 was used to lyse cells that remained attached to the plates. When cells lyse they will release neutral red which can be analyzed for amount using a spectrophotometer. The positive control was gliotoxin of different amounts.

Results

In general, there was a weak trend showing higher absorbance for the clinical isolates. HPLC results and calculations of the amount of gliotoxin in the samples in micrograms using standard curve equation are as follows:

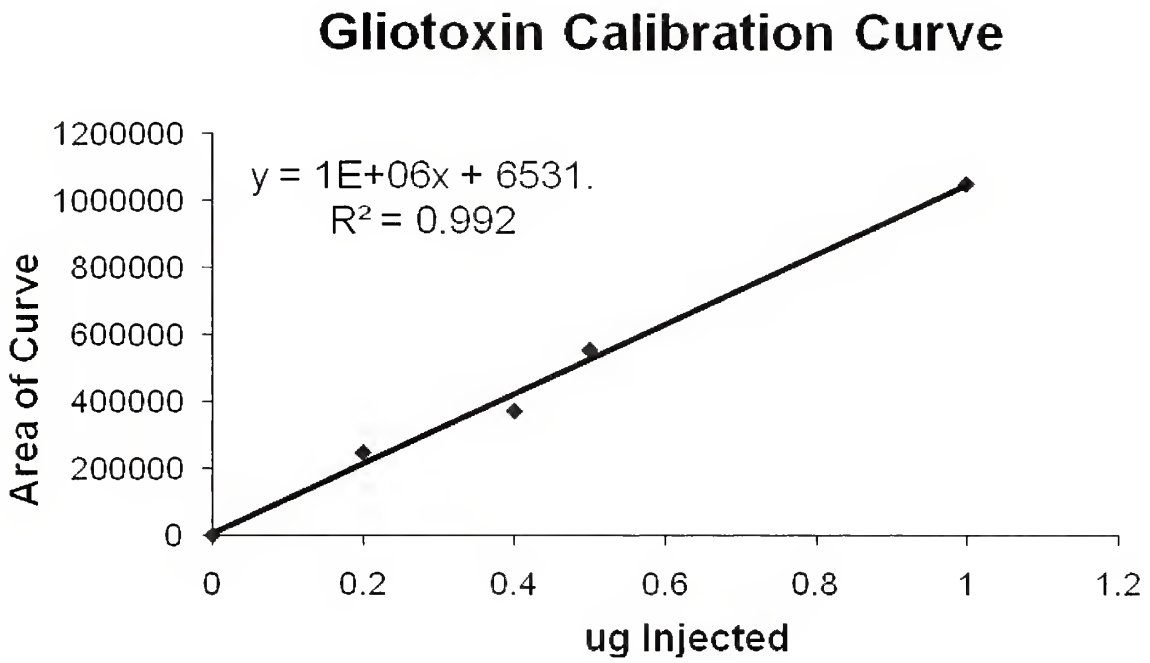
Table 2. Amount of gliotoxin in cell extract by chloroform

Sample	Area	ug in Culture Tube
5215	9787	0.32559
5216	37064	3.05329
5267	184	0
5854	68887	6.23559
5211	4018	0
5262	0	0
5217	0	0
5263	0	0
5214	0	0

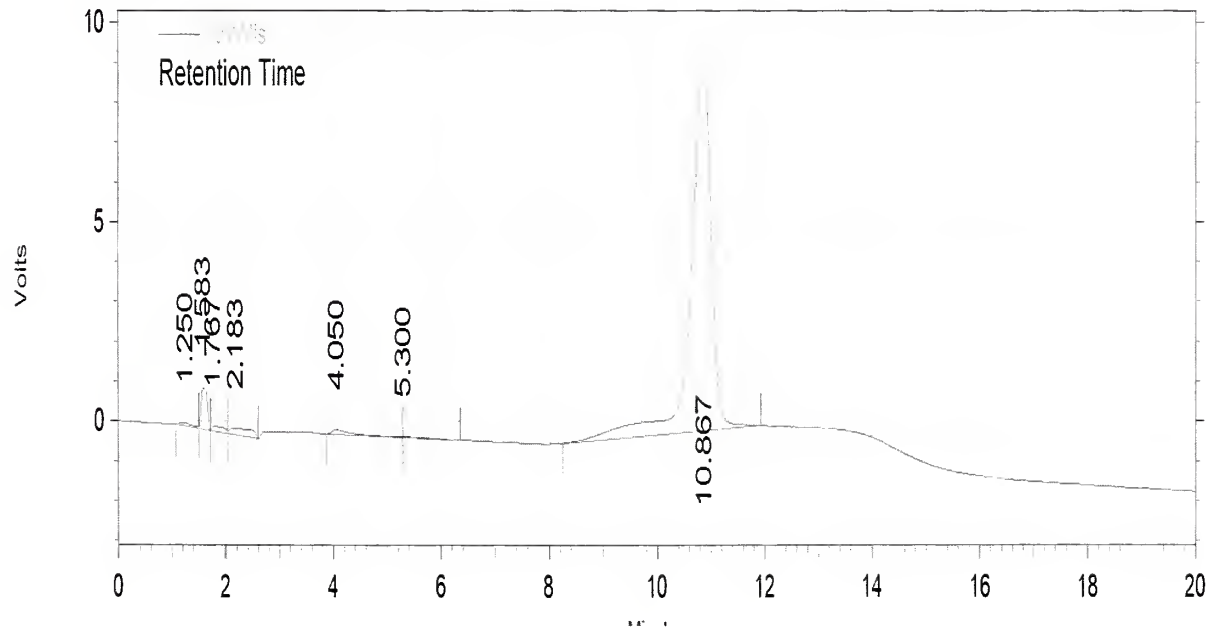
Table 3. Gliotoxin standardization curve

Dilution Injected (ug)	Area of Peak
10	7567317
1	1052354
0.5	554641
0.4	371297
0.2	246826

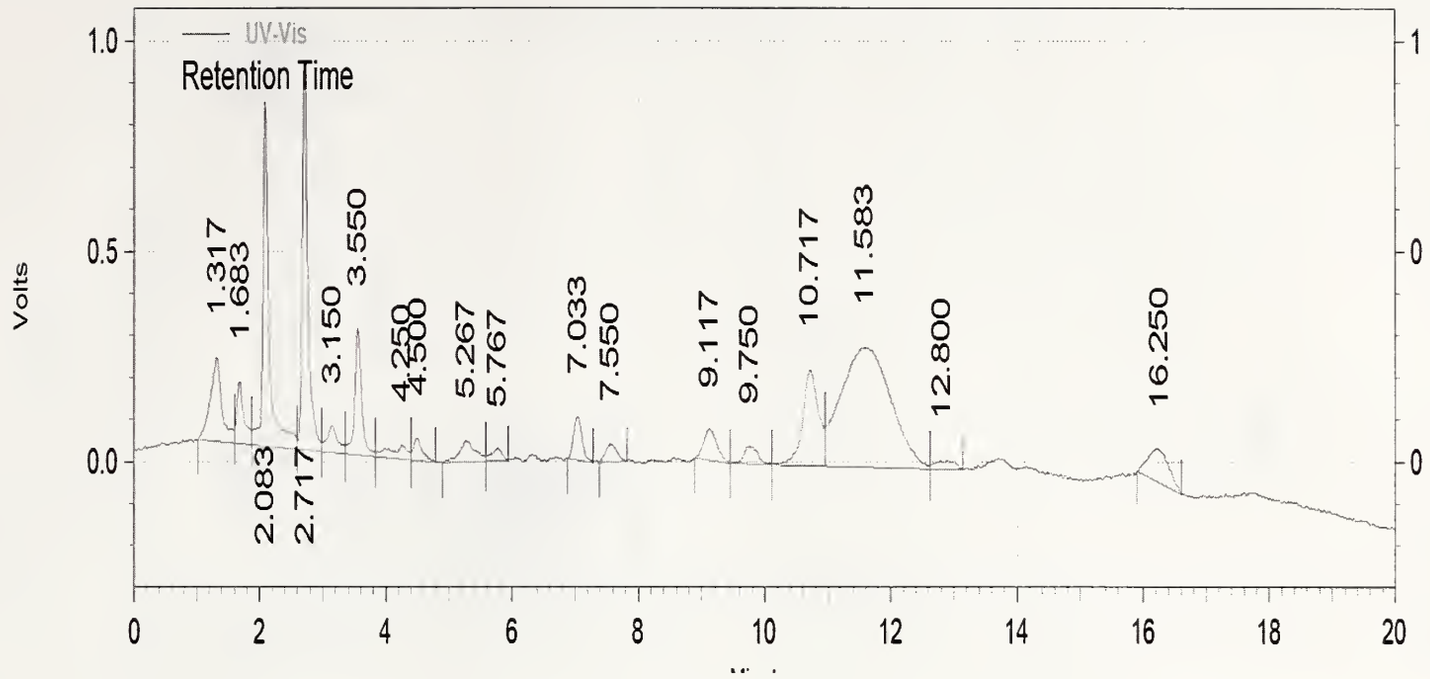
Figure 1. Gliotoxin Calibration Curve



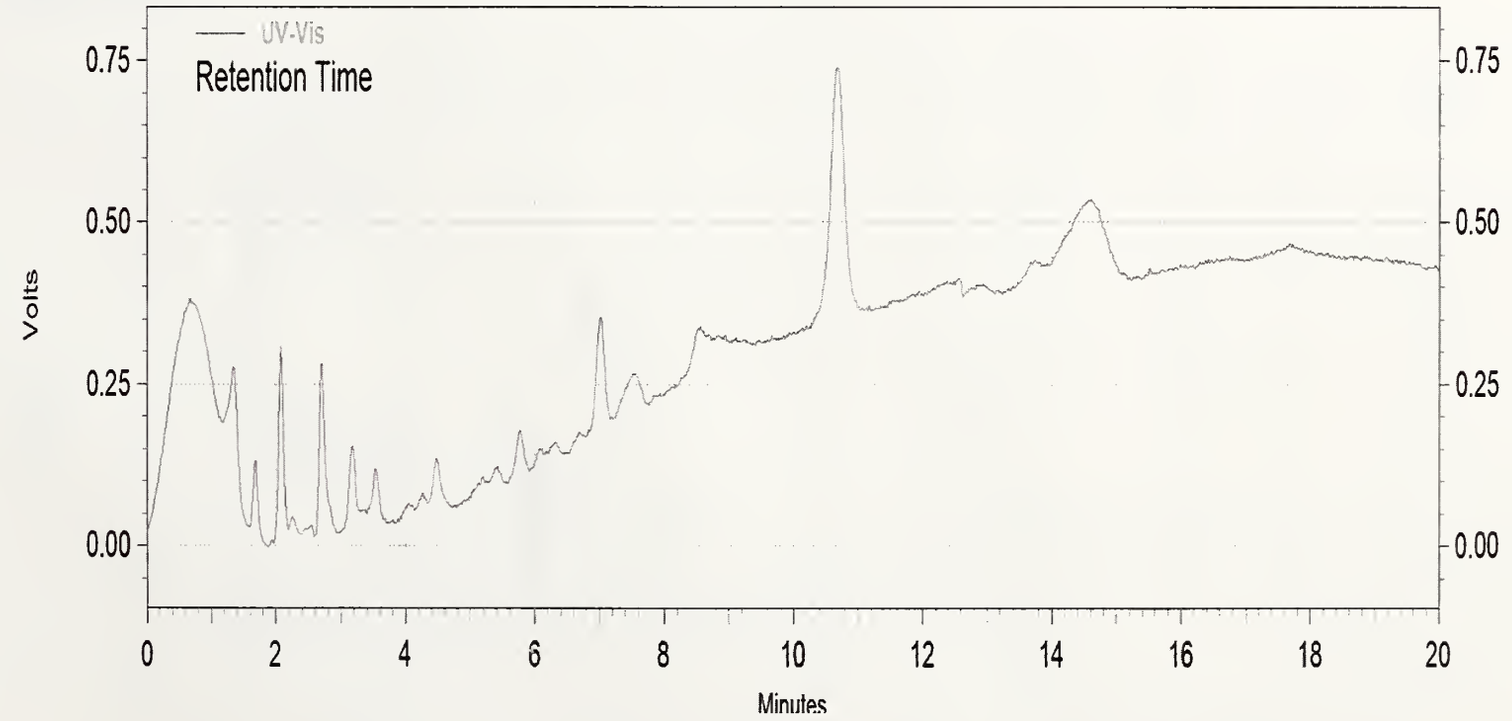
HPLC Graphs



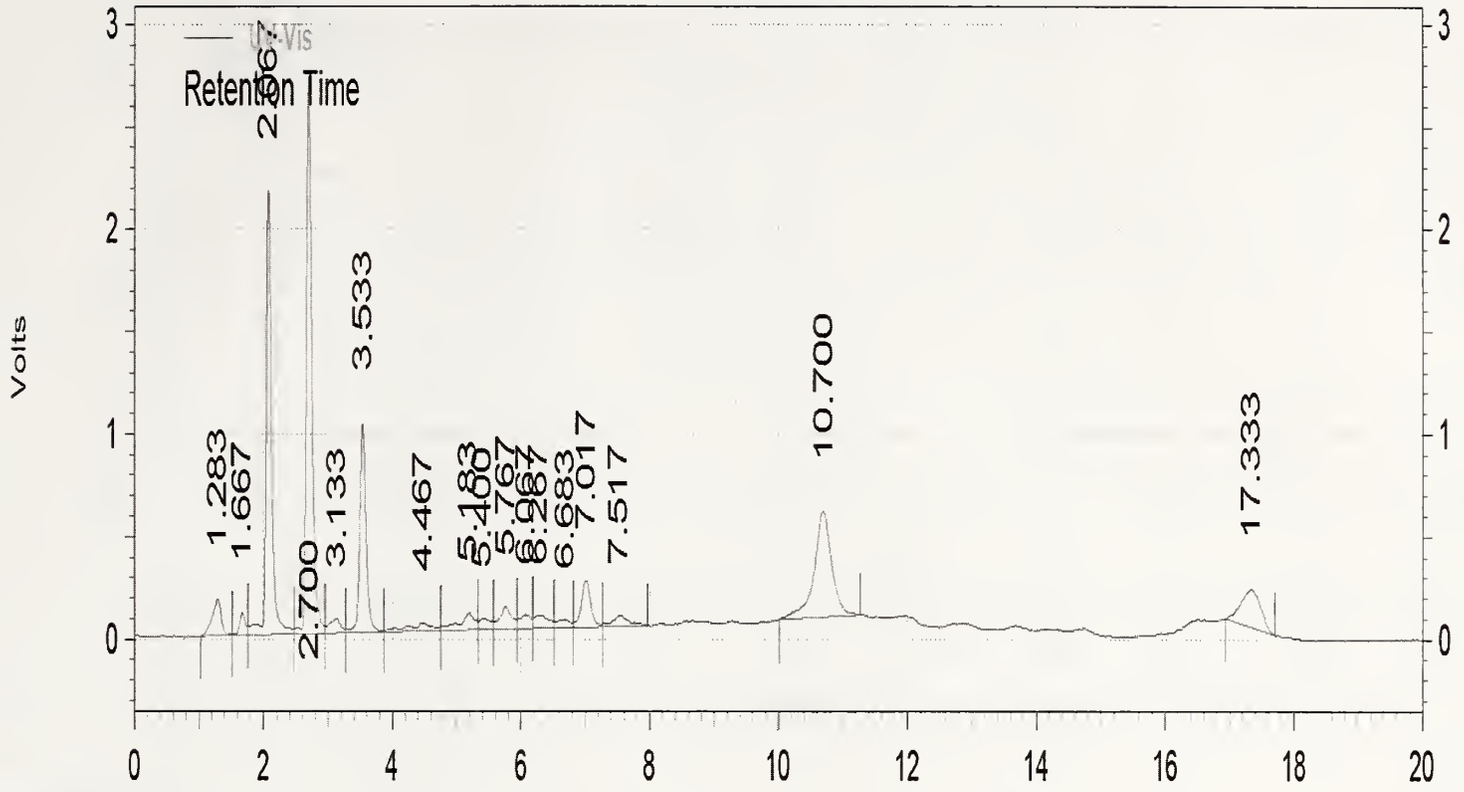
0.2ug standard curve dilution (lowest dilution)



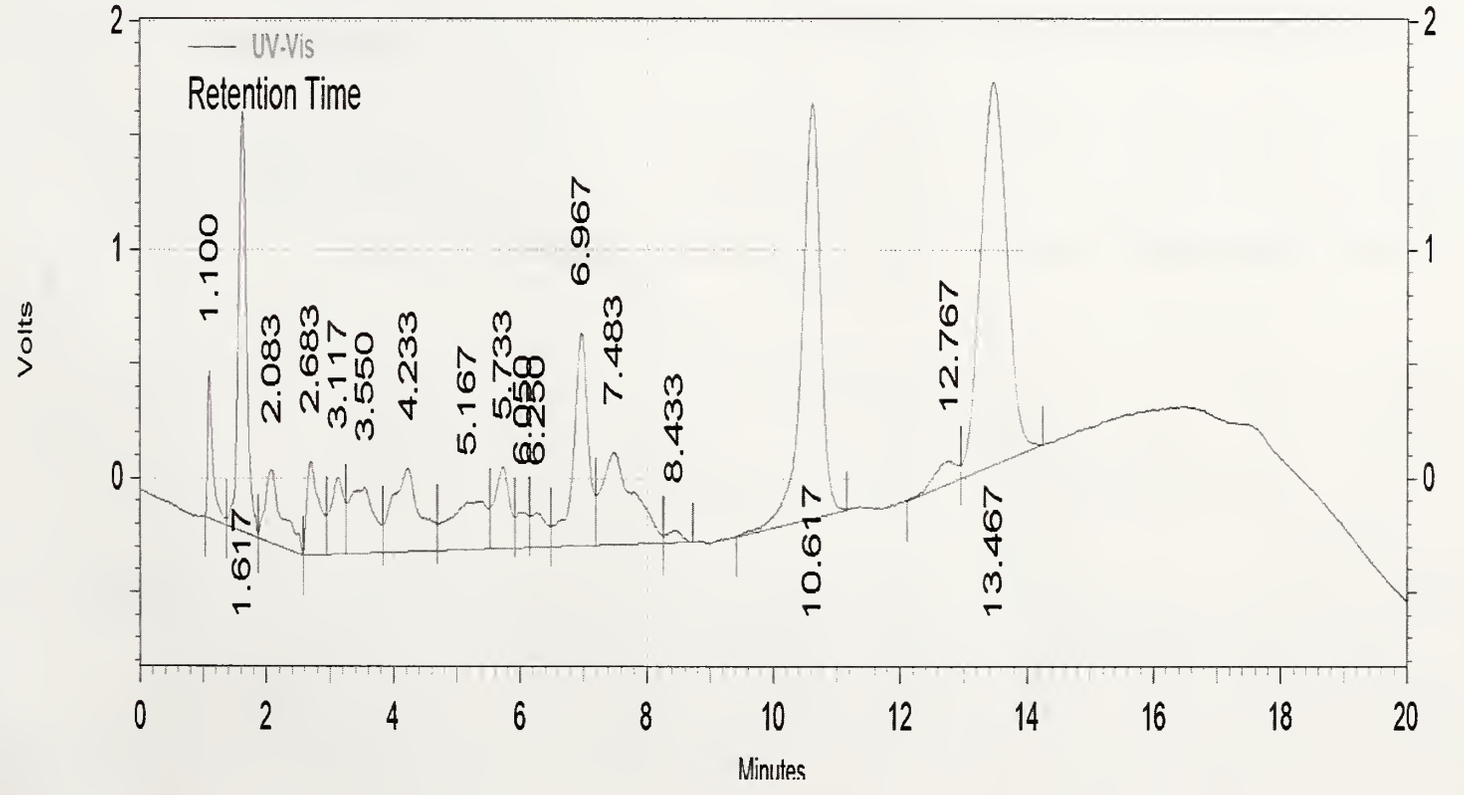
Sample 5211



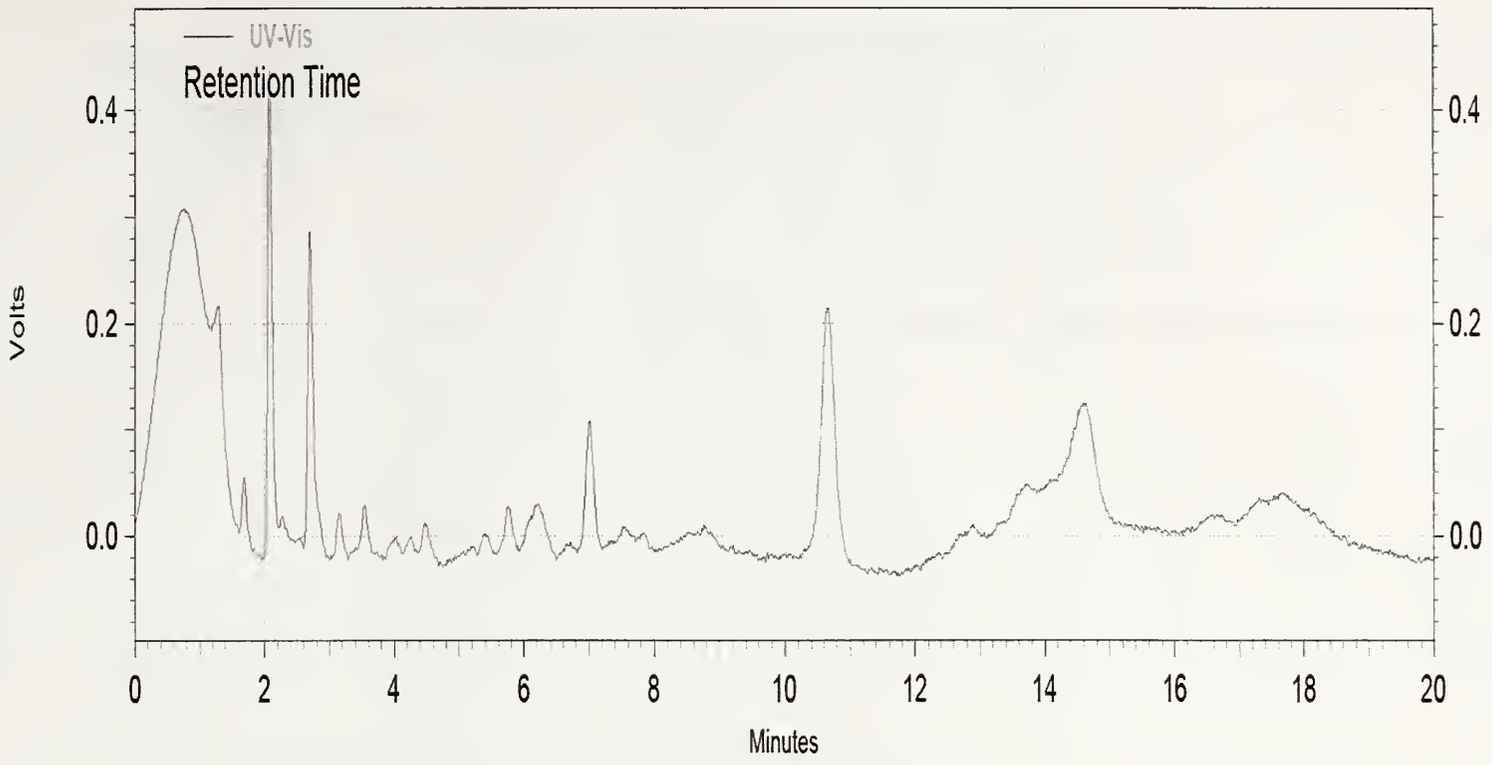
Sample 5214



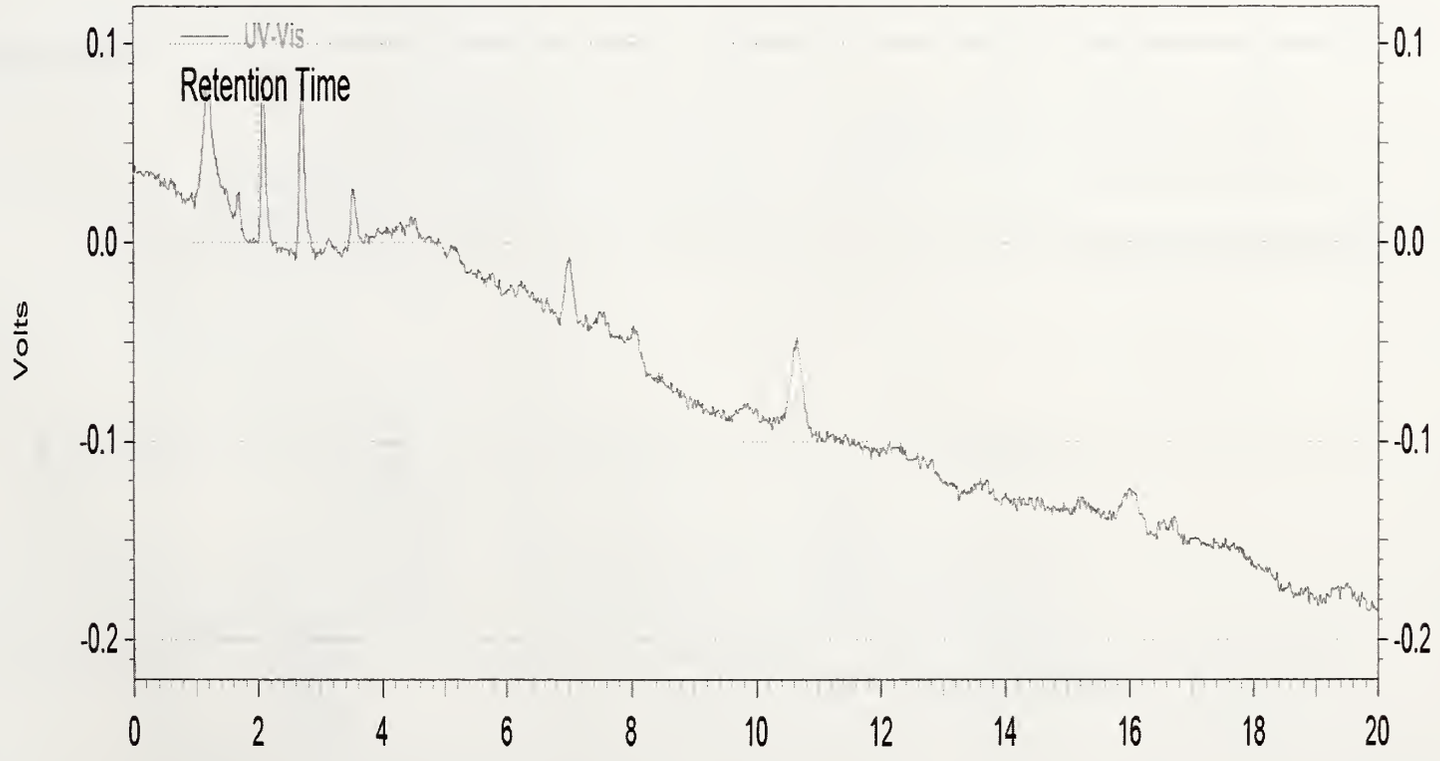
Sample 5215



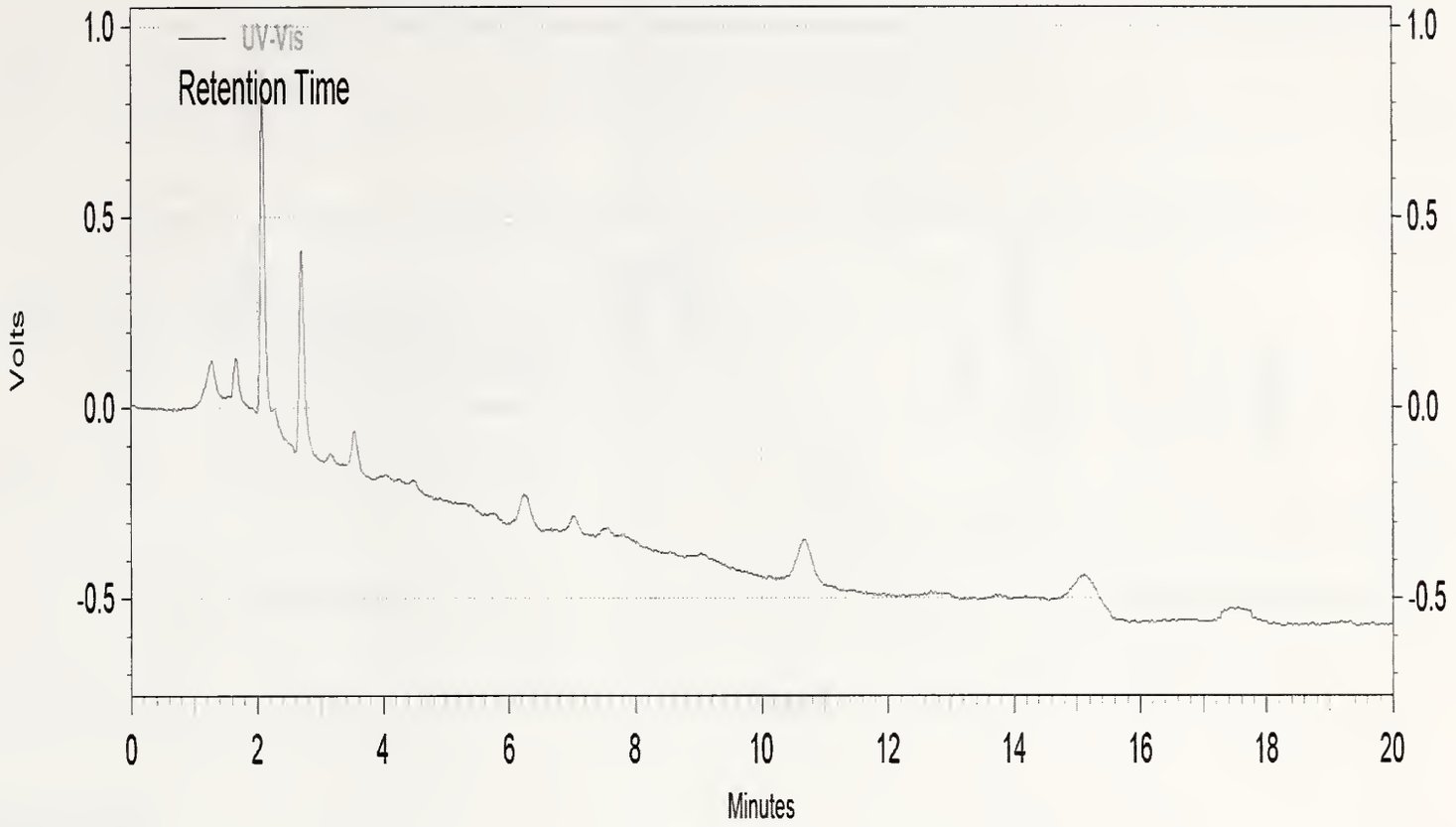
Sample 5216



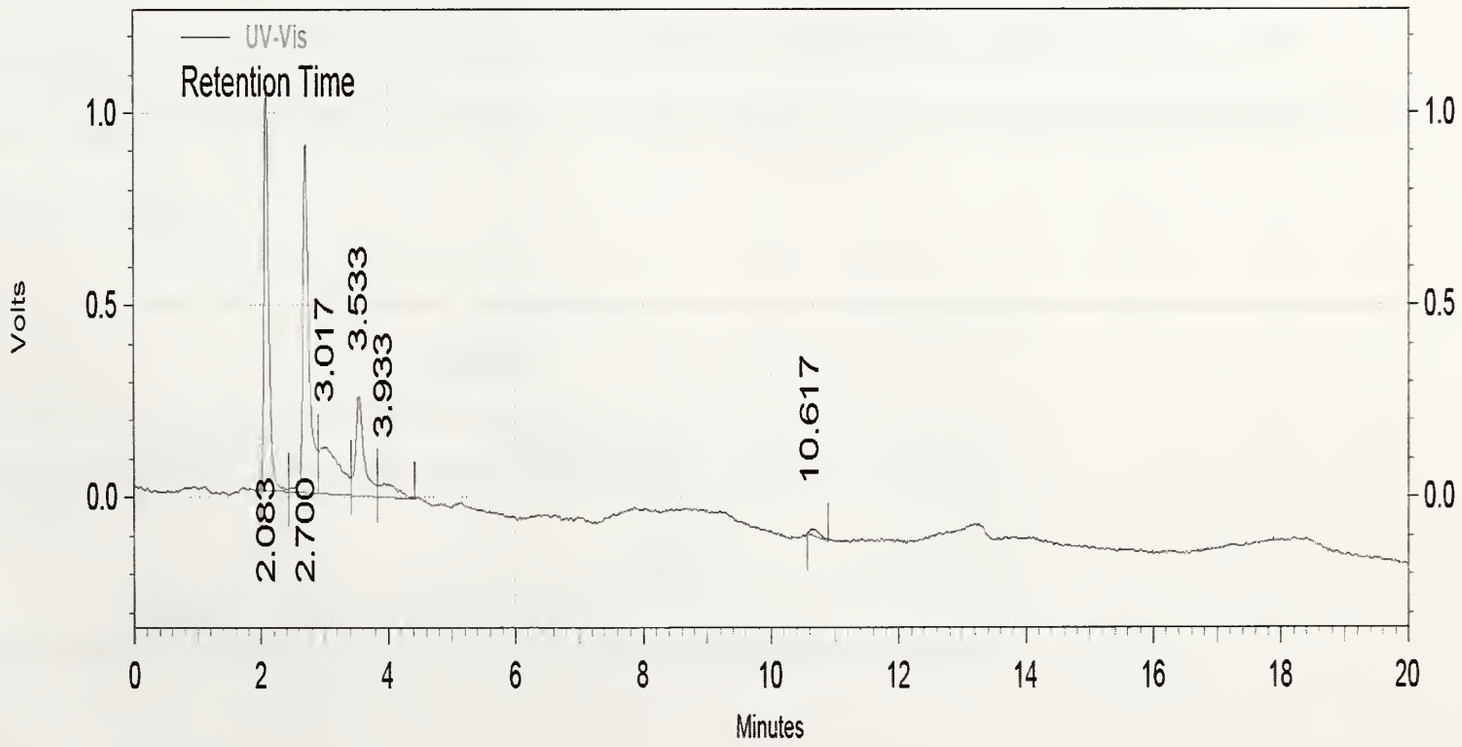
Sample 5217



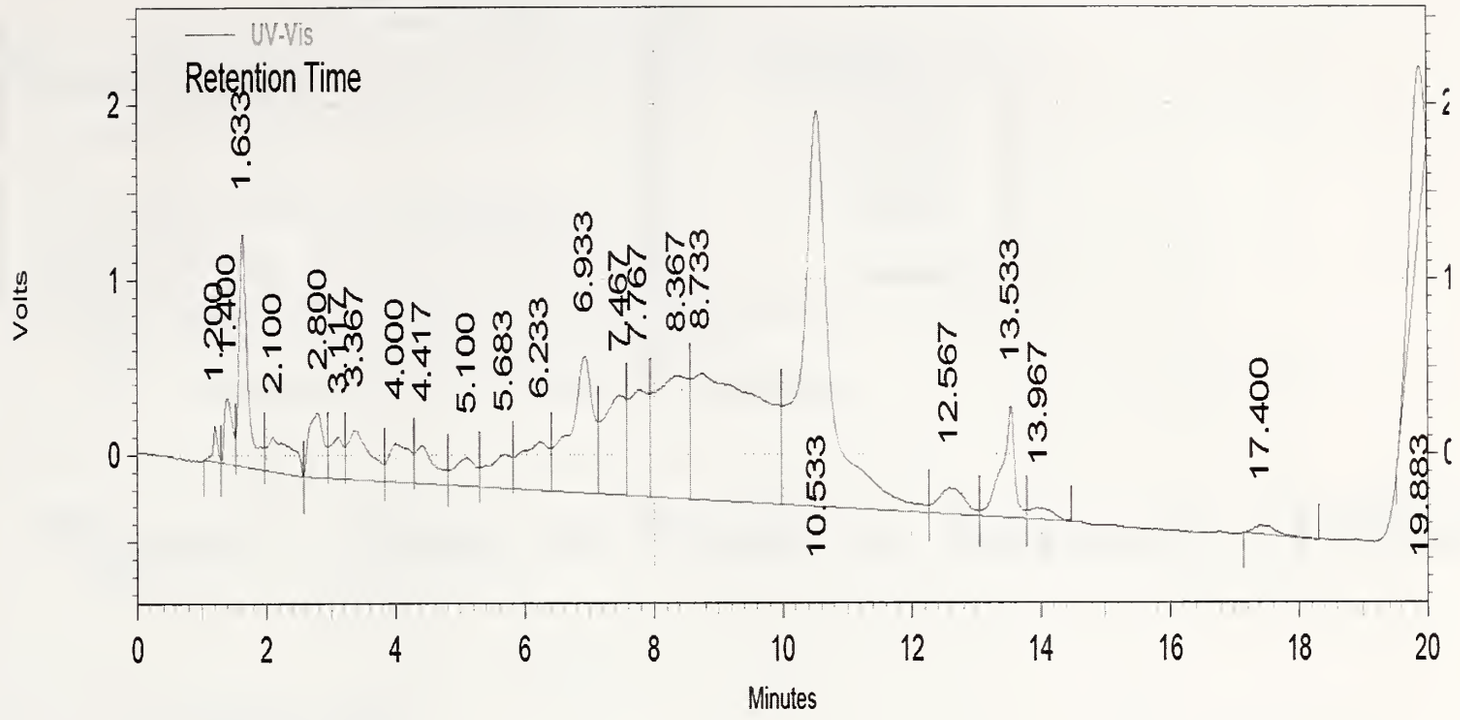
Sample 5262



Sample 5263



Sample 5267



Sample 5854

We used Whitney Mann non-parametric test to check the significance between peak areas of clinical and environmental isolates. Non-parametric tests use rankings instead of numbers. Unlike parametric tests such as ANOVA, they do not assume a normal distribution. Results showed that the amount of gliotoxin produced by the clinical and environmental isolates was not significantly different (W=22.5, P= 0.556).

Ranks

	Sample	N	Mean Rank	Sum of Ranks
Area	1	5	4.50	22.50
	2	4	5.63	22.50
	Total	9		

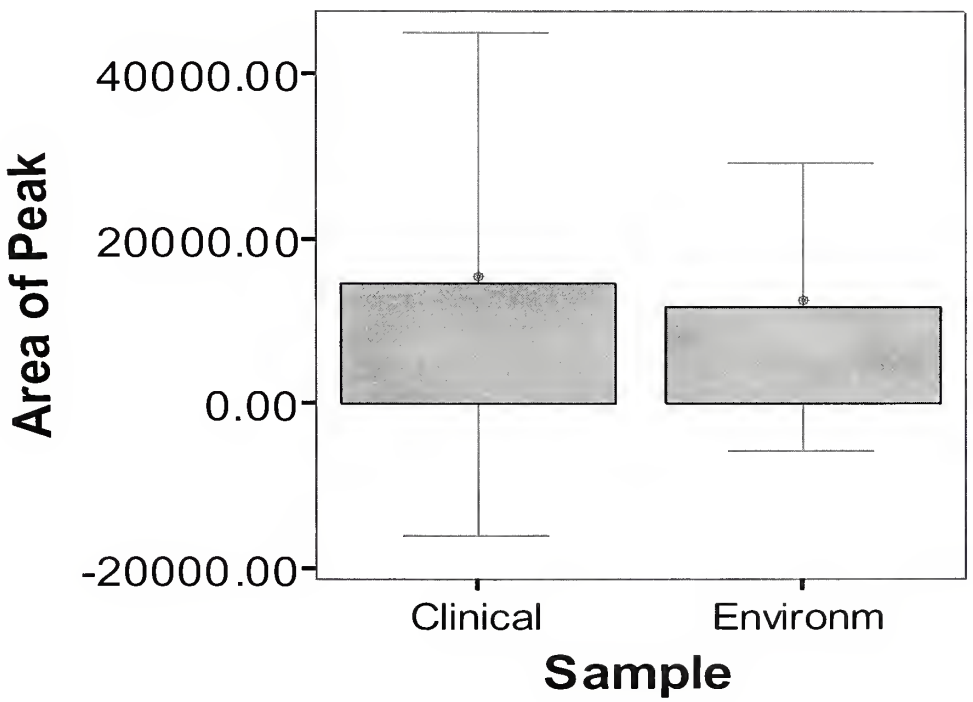
Test Statistics[b]

	Area
Mann-Whitney U	7.500
Wilcoxon W	22.500
Z	-.640
Asymp. Sig. (2-tailed)	.522
Exact Sig. [2*(1-tailed Sig.)]	.556[a]

a. Not corrected for ties.

b. Grouping Variable: Sample

Figure 1. Area of Peak vs. Amount of Glioto



Discussion

Using standard dilutions of synthetic gliotoxin, we consistently found a peak at eleven minutes for the five dilutions. When making the standard curve one dilution was omitted because it was an outlier. The eleven minute peak has previously been found to indicate the presence of gliotoxin. None of the samples, whether clinical or environmental, produced peaks areas close to the area of the

lowest dilution of synthetic gliotoxin, 0.2ug. This was chosen as the lowest dilution because gliotoxin shows toxic effects at concentrations as low as 0.03-1ug/ml. All the calculated values for the amount of gliotoxin in the samples were less that 0.2ug. The clinical isolates were labeled 5211, 5214, 5262, 5263, and 5854. The environmental isolates were 5215, 5216, 5217, and 5267. Three of the environmental isolates produced a peak while two of the clinical isolates produced a peak (Table 2). The standard curve equation was used to estimate the amount of gliotoxin in each extract. Most of the calculated values for the amount of gliotoxin in the samples showed that the amount of gliotoxin was very low or absent.

Scientists could extend this study by having a higher number of samples for the trials. More samples could be used to note trends within the clinical and environmental isolates. Since a majority of the samples did not produce a peak for the gliotoxin, maybe a more sensitive method should be used. Gliotoxin may be the virulence factor but results from this study do not adequately support our hypothesis that the amounts of gliotoxin would be higher in the clinical isolates of *A. fumigatus.* It might be that gliotoxin is a major virulence factor when combined with the other compounds released during growth of the mold. The effects of other compounds such as ribonuclease and haemolysin might combine with the effects of gliotoxin to produce the serious symptoms of aspergillosis.

Bibliography

Amitani, Ryoichi, G. Taylor, E. Elezis, C. Llewellyn-Jones, J. Mitchell, F. Kuze, P.J. Cole and R. Wilson. 1995. Purification and characterization of factors produced by *Aspergillus fumigatus* which affect ciliated respiratory epithelium. Infection and Immunity. 63(9): 3266-3271.

Bernard E. Tuch, J. R. Lissing, and M.G. Suranyi. 1988. Immunodulation of Human Fetal Cells by the Fungal Metabolite Gliotoxin. Immunology and Cell Biology. 66: 307-312.

Bignell, Elaine M, G.H. Braus, K. Haynes, S. Krappmann, U. Reichard and T. Rogers. 2004. The *Aspergillus fumigatus* Transcriptional Activator CpCA Contributes Significantly to the Virulence of this Fungal Pathogen. Molecular Microbiology. 52(3): 785-799.

Jean-Paul Latge. 1999. *Aspergillus fumigatus* and Aspergillosis. Clinical Microbiology Reviews. 12(2): 310-350.

Jean-Paul Latge. 2001. The Pathobiology of *Aspergillus fumigatus*. Trends in Microbiology. 9(8): 382-389.

Jin Woo Bok, D. Chung, S.A. Balajee, K.A. Marr, D. Andes, K.F. Nielsen, J.C. Frisvad, K.A. Kirby, and N. P. Keller. 2006. GliZ, a Transcriptional Regulator of Gliotoxin Byosnthesis, Contributes to *Aspergillus fumigatus* Virulence. 74 (12): 6761-6768.

J.L. Richard, R.L. Lyon, R.E. Fichner, and P.F. Ross. 1989. Use of Thin Layer Chromatography for Detection and High Performance Liquid Chromatography for Quantitating Gliotoxin from Rice Cultures of Aspergillus fumigatus Fresenius. Mycopathologia. 107: 145-151.

Kauffmann H.F. and J.F. CH. Tommee. 2000. Putative virulence factors of *Aspergillus fumigatus*. Clinical and Experimental Allergy. 30: 476-484.

Philip Sutton, P. Waring, and A. Müllbacher. 1996. Exacerbation of Invasive Aspergillosis by the Immunosuppressive Fungal Metabolite, Gliotoxin. Immunology and Cell Biology. 74:318-322.

Singh, Nina and D. L. Paterson. 2005. Aspergillus Infections in Transplant Recipients. American Society for Microbiology. 18(1): 44-69.

Acknowledgements

Dr. James Simon – HPLC

The Columbus State University Honors Program